

**HIGH TRANSMISSION OF PATERNAL PLASTID DNA IN ALFALFA PLANTS
AS SHOWN BY RESTRICTION POLYMORPHIC ANALYSIS**

by

SAMEER AHMAD MASOUD

B.S., University of Jordan
Amman, Jordan, 1982

A MASTER'S THESIS

submitted in partial fulfillment of the
requirements for the degree

MASTER OF SCIENCE

Department of Plant Pathology

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1989

Approved by:

Lowell B. Johnson

Major Professor

ACKNOWLEDGEMENTS

11208 304425

I wish to express sincere appreciation to my major advisor, Dr. Lowell Johnson, for his suggestions, patient guidance, and friendship throughout the course of this investigation and preparation of this thesis.

Gratitude is extended to Dr. Frank white for use of laboratory equipment, suggestions, and for serving on the advisory committee.

Appreciation is extended to Dr. Edgar Sorensen for helping start the research by assisting with the interploidy crosses, for providing some parental plant material, as well as for serving as a member of the advisory committee.

Warm thanks is given to Dr. Mark Thomas, post-doctorate, for his help, suggestions, and useful discussion.

Gratitude is extended to the department of plant pathology for the Graduate Research Assistantship which helped make my graduate study possible.

Appreciation is extended to Dr. Jeffrey Palmer for providing cloned alfalfa chloroplast DNA, Dr. James Schwenke for helping in statistical analysis, and Reneé Hart for her help and encouragement.

Special appreciation is extended to my parents, Mr. and Mrs. Ahmad Masoud, and family for their constant encouragement and support during the entire study at Kansas State University.

TO

MY PARENTS

TABLE OF CONTENTS

Acknowledgements.....	i
Dedication.....	ii
Table of contents.....	iii
List of Tables and Figures.....	iv
 I CHAPTER ONE: LITERATURE REVIEW OF BIPARENTAL INHERITANCE OF CHLOROPLASTS IN ALFALFA: A COMPARISON WITH PLASTID TRANSMISSION IN OTHER SPECIES	
1) INTRODUCTION.....	1
2) MECHANISMS OF PATERNAL PLASTID ELIMINATION IN SEXUAL REPRODUCTION.....	4
3) MECHANISMS OF BIPARENTAL PLASTID INHERITANCE.....	14
4) GENETIC CONTROL OF PLASTID INHERITANCE IN SEXUAL REPRODUCTION.....	19
5) CHLOROPLAST INHERITANCE IN ALFALFA.....	23
6) REFERENCES.....	27
 II CHAPTER TWO: HIGH TRANSMISSION OF PATERNAL PLASTID DNA IN ALFALFA PLANTS AS SHOWN BY RESTRICTION POLYMORPHIC ANALYSIS	
1) INTRODUCTION.....	32
2) MATERIALS AND METHODS.....	34
3) RESULTS.....	39
4) DISCUSSION.....	42
5) REFERENCES.....	48
6) TABLES AND FIGURES.....	51

LIST OF TABLES AND FIGURES

Table 1. Restriction fragment length polymorphisms (in kbp) used to distinguish parental ctDNAs following restriction enzyme digestion and hybridization with alfalfa ctDNA fragments.....	51
Table 2. Single and reciprocal crosses used for evaluating ctDNA transmission of different alfalfa genotypes.....	52
Table 3. Summary of crosses within and between two <u>Medicago sativa</u> subspecies.....	53
Fig. 1. A schematic representation of all sexual crosses made for evaluating paternal plastid transmission.....	54
Fig. 2A-D. Autoradiograms showing ctDNA transmission in alfalfa sexual crosses, and conversion of heteroplasmy to homoplasmy during vegetative propagation.....	55

CHAPTER ONE:
LITERATURE REVIEW OF
BIPARENTAL INHERITANCE OF CHLOROPLASTS IN ALFALFA:
A COMPARISON WITH PLASTID TRANSMISSION IN OTHER SPECIES

INTRODUCTION

The chloroplast is a unique autonomous organelle with non-Mendelian inheritance. Its non-Mendelian transmission, as observed using chlorophyll deficiency traits, was first described independently in 1909 by Correns in four-o'clock (Mirabilis jalapa) and by Baur in garden geranium (Pelargonium zonale), shortly after the rediscovery of Mendel's laws of inheritance (Gillham 1978). They observed maternal and biparental inheritance patterns, respectively.

Transmission of genetic information through extranuclear particles shows the following characteristics: 1) transmission does not follow Mendelian segregation ratios for a given cross; 2) ratios vary with the direction of the cross; and 3) the characters segregate during somatic development and clonal growth of hybrids (Cornu and Dulieu 1988). About one-third of the approximately 60 higher plant genera studied are classified as having biparental plastid transmission (Kirk and Tilney-Bassett 1978; Gillham 1978; Sears 1980).

The circular plastid genome (ctDNA) encodes part of the gene products that are involved in chloroplast biogenesis.

These include the large subunits of ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco), and only 19 of 52 of the plastid ribosomal proteins (Mullet 1988). Other plastid proteins are nuclear encoded. Nuclear DNA also regulates ctDNA gene expression (Daday et al. 1987). The level of Rubisco in the chloroplast is controlled by the cell nucleus which encodes for the small subunits of the enzyme (Daday et al. 1987). Moreover, plastids are necessary for activation of some nuclear genes involved in plastidogenesis (Oelmüller et al. 1986). A short-lived signal released by the plastids activates the expression of both the Rubisco small subunit and the light-harvesting chlorophyll a/b-binding protein of photosystem II (Oelmüller et al. 1986).

Much research effort is currently being directed toward a better understanding of organelle inheritance in higher plants, due in part to the availability of new techniques and potential applications. One such technique is the use of restriction patterns of ctDNA, which allows studying types and frequencies of plastid transmission. Biparental plastid inheritance was demonstrated in several plant species by using this technique (Szmidt et al. 1987; Wagner et al. 1987; Lee et al. 1988). Biparental inheritance was reported in alfalfa following studies with plastid-encoded mutants for chlorophyll-deficiency (Smith et al. 1986) and ctDNA polymorphisms (Lee et al. 1988).

The use of ctDNA as a marker for plastid inheritance may

allow a more meaningful estimation of the frequency of transmission than does the use of chlorophyll deficiency mutants, which may be at a competitive disadvantage in plastid transmission due to the mutation. In Pelargonium, green plastids were transmitted at a higher rate than mutant plastids (Tilney-Bassett and Birky 1981; Tilney-Bassett and Abdel-Wahab 1982). Two chloroplast mutants of Oenothera exhibited reduced transmission from the female parent relative to a third mutant, which was transmitted similarly to the wild type (Chiu et al. 1988).

There are other advantages as well for using restriction fragment length polymorphisms of ctDNA. The presence of suitable polymorphic ctDNA markers allows a larger number of different reciprocal crosses to be analyzed for their plastid transmission, yet still retains a high sensitivity for detection of a minor plastid population in a green heteroplastidic tissue. Reciprocal crosses are not limited to plant genotypes that differ in plastid-encoded chlorophyll deficiency mutants. Using ctDNA markers also allows identification of all plastid types (including proplastids). Aberrant transmission ratios due to seedling death resulting from chlorophyll deficiency mutants are eliminated. Induction and reversion of plastid mutation by nuclear mutator genes are common in plants and may interfere with the analysis of chloroplast inheritance when chlorophyll deficiency markers are used (Gillham 1978; Cornu and Dulieu 1988). These

mutations are rarely seen in restriction enzyme analysis. Comparative studies of plastid transmission in which relative amounts of both chlorophyll deficiency and ctDNA restriction pattern markers are followed simultaneously are not available.

Other techniques have been used to detail the cellular events that occur before and after fertilization. These include the use of serial ultra-thin sections and computer-generated three-dimensional image reconstructions (Mogensen 1988; Mogensen and Wagner 1987; Dumas et al. 1984). However, ctDNA is the genetic material that is transmitted from parent to progeny and electron microscopic observation of plastids is inadequate evidence for the presence of their DNA (Miyamura et al. 1987). ct-Nucleoid staining by 4'-6-diamidino-2-phenylindole (DAPI) and anti-DNA antibody were used to follow the fate of ctDNA during male gamete development and chloroplast dedifferentiation (Kuroiwa and Hori 1986; Kuroiwa et al. 1988).

A better understanding of plastid inheritance may permit an improved assessment of the potential agronomic significance of ctDNA. This includes attempts to obtain novel combinations of nuclear and cytoplasmic genomes in cybrids following protoplast fusion, and efforts to modify the plastid genome by chloroplast microinjection or transformation.

MECHANISMS OF PATERNAL PLASTID ELIMINATION IN SEXUAL REPRODUCTION

Several hypotheses have been proposed to explain mechanisms of paternal plastid elimination in higher plants. Paternal transmission may result from an absence of these mechanisms. In species where chloroplast transmission is maternal, elimination of paternal plastids (Connett 1987) or their DNA (Miyamura et al. 1987) may occur at any developmental stage between spermatogenesis and postfertilization. The diverse ways in which maternal plastid inheritance is achieved suggest that this inheritance pattern evolved independently many times in different taxonomic groups during evolution in response to different selective pressures (Sears 1980). The relative evolutionary advantages of biparental or uniparental plastid inheritance are unclear.

Early workers assumed that the sole mechanisms of maternal plastid transmission were the physical exclusion of the ctDNA from the male generative cell or the degeneration of plastids during pollen maturation (Connett 1987; Chiu et al. 1988). However, physical exclusion alone can not explain the maternal transmission observed in plant species where paternal plastids occur in the zygote and yet maternal inheritance follows (Gillham et al. 1987; Kuroiwa 1985).

If the paternal plastids survive elimination, hetroplastidic tissue is produced. This hetroplastidic condition usually results in complete or partial sorting of the plastids in different parts of the plant to produce two

types of homoplastidic cells (Kirk and Tilney-Bassett 1978; Gillham 1978; Birky 1983). Similar sorting occurs in somatic hybrids following fusion of protoplasts from two plants having different chloroplast types (Fluhr 1983). Somatic hybrid plants may show either random (D'hont et al. 1987) or biased (Fluhr 1983) chloroplast transmission.

In a classification modified after Sears (1980), mechanisms of maternal inheritance in plants can be placed into three major categories based on timing of plastid elimination; prefertilization, during fertilization, and postfertilization. Maternal plastid inheritance occurs whenever paternal plastids or their DNA are eliminated during one of these stages:

Exclusion of plastids or their DNA before fertilization

It is commonly thought that maternal inheritance of cytoplasmic characters results because the male gamete contains little cytoplasm and few or no organelles. The absence of plastids from generative cells was reported in several plant species (Miyamura et al. 1987; Corriveau and Coleman 1988). However, small proplastids clustered inside the generative cells may be difficult to identify, possibly explaining their reported absence in several plant species (Connett 1987). Plastid loss from the male gamete may occur in plants during spermatogenesis or during pollen tube development. Losses at either time might provide a basis for

chloroplast maternal inheritance in plants (Connett 1987).

Both chloroplasts and the cell cytoskeleton function in establishing polarity during spore formation in mosses. Meiosis results in the production of four spores, each with a single nucleus and plastid. Plastid dedifferentiation and reduction in number per cell occur early in sporogenesis (Busby and Gunning 1988).

In angiosperms, the first mitosis in microspore formation is polar, and produces unequal cells (Tanaka 1988). The smaller generative cell ultimately gives rise to a pair of sperm nuclei. Polarity is observed in wheat when unequal division of the monocellular pollen grain produces a large vegetative cell and a small generative cell which at this stage still stains strongly for plastid nucleoids. In other species such as Easter lily and tobacco, generative cells are already negative for plastid nucleoids (Miyamura et al. 1987).

Both mitochondria and plastids occur in the early generative cells of Solanum. During maturation, vegetative cells showed little change in the contents of these organelles, while generative cells retained mitochondria but completely lost their plastids (Clauhs and Grun 1977). A selective loss of plastids or their DNA during gametogenesis could result in maternal plastid transmission (Kuroiwa et al. 1988).

Paternal plastids at any developmental stage of the male gamete might lack DNA, which would lead to maternal plastid

inheritance. Electron microscopic observation does not reveal plastid nucleoids. Preferential destruction of the male ctDNA during pollen maturation and before gamete fusion was shown in algae such as Bryopsis maxima (Kuroiwa and Hori 1986) and the fern, Pteris vittata L. (Kuroiwa et al. 1988). Destruction of the paternal chloroplast nucleoid is associated with a remarkable decrease in volume and DNA content of the chloroplasts, which differentiate into amyloplasts (Kuroiwa et al. 1988). Plastids are the only cell organelles that store starch, which may serve as a food source for lower plant gametes (Sears 1980).

The maternal inheritance in some fern species apparently results from the loss of the whole cytoplasmic vesicle prior to or during fertilization (Kuroiwa et al. 1988). Male plastid nucleoids disappeared during spermatogenesis in Chara corallina, as judged by staining with the DNA-specific fluorochrome DAPI and with monoclonal anti-DNA antibody (Sun et al. 1988). This results in maternal plastid inheritance in the Characeae, a family which probably occupies a taxonomic position linking green algae and land plants (Sun et al. 1988).

Vaughn et al. (1981) hypothesized that organelle alteration in the pollen explains maternal inheritance in higher plants, with paternal plastid alteration being the first step of plastid elimination. They reported that a severely debilitated plastome mutant, although present in pollen

generative cells, was not transmitted by pollen of Pelargonium, which usually exhibits biparental plastid inheritance (Vaughn 1981). Cornu and Dulieu (1988) concluded that even in plants such as Petunia that typically exhibit maternal plastid inheritance, variant nuclear genes could impair the normal mechanism of pollen plastome elimination, presumably by protecting the ctDNA during microsporogenesis and pollen differentiation.

Albino wheat plants occur commonly following regeneration from pollen culture, and have large deletions in their ctDNA (Day and Ellis 1984). The frequency of chlorophyll-deficient plants from pollen culture increases when pollen is more mature. This suggests a possible mechanism for maternal inheritance in wheat (Day and Ellis 1984). Miyamura et al. (1987) provided additional evidence for the role of ctDNA breakdown in maternal inheritance in wheat, using fluorescence microscopy to study pollen grain formation and development. Plastid nucleoids changed morphologically during early stages of pollen formation, and ultimately disappeared during pollen tube maturation. In contrast, seven out of fifteen other plant species tested had ct-nucleoids in their mature pollen generative cells. Two of these seven are reported to have biparental plastid inheritance (Miyamura et al. 1987). Corriveau and Coleman (1988) used this principle to screen rapidly for higher plant species potentially capable of biparental inheritance. Pollen from 235 plant species

representing 80 families was screened. Plastid DNA was detected in the generative and/or sperm cells of pollen from 43 species, most of which are thus presumed to exhibit biparental plastid inheritance.

Exclusion of plastids during fertilization

The actual events of fertilization in higher plants are poorly understood because of the difficulties in conducting such studies (Dumas et al. 1984). The timing of cytological observations between pollination and fertilization is difficult to predict. The time of arrival of the pollen tube to the embryo sac is variable and is rapidly followed by fertilization. Part of the cytoplasmic contents of the pollen tube may discharge into the synergid before the sperm cell fuses with the egg (Mogensen 1982, 1988; Sears 1980; Connett 1987).

The two male gametes of common origin, along with the vegetative nucleus, comprise a closely associated unit that is maintained in flowering plants throughout pollen tube growth (Dumas et al. 1984; Mogensen and Wagner 1987). This unit contains both the male nuclear and cytoplasmic DNAs. Flowering plant reproduction requires two independent fertilization events, i. e. double fertilization, which is directed and not random (Dumas et al. 1984; Russell 1987). Each sperm cell fuses with a different female cell within the

embryo sac of the ovule (future seed) in an ordered manner. One pollen sperm cell fertilizes the egg to form a diploid zygote. The second sperm cell fuses with the central cell nucleus to form a triploid endosperm (Dumas et al. 1984; Goldberg 1988).

The male gametes of cotton and Petunia have been studied during all postpollination stages from pollen activation through pollen tube growth to the ovule (Mogensen 1982). Pollen cytoplasm apparently remains in the degenerating synergid, with only the sperm nucleus entering the egg. This also occurs in barley, and apparently results in maternal inheritance of plastids, which are present in the male pollen grains (Mogensen 1982, 1988). The sperm nucleus within the egg of barley loses its cytoplasmic sheath, which is present in the intracellular space outside the zygote at the point where the sperm nucleus entered. Observation by electron microscopy suggests that the elimination of paternal plastids of barley occurs during syngamy (Mogensen 1982, 1988). Interestingly, ct-nucleoids were not detected in the generative cells of barley, cotton, and Petunia when their pollen was stained by DAPI and observed under fluorescence microscopy (Corriveau and Coleman 1988).

Exclusion of plastids after fertilization

A species that transmits its plastids in the pollen tube and

does not shed them during syngamy will have plastids from both parents in the fertilized egg. Comparable somatic hybrid cells usually contain two types of plastids after protoplast fusion. They may sort out in either a random or a biased pattern (Fluhr 1983), but I have seen no reports of biased maternal sorting out of plastids in higher plant sexual embryos.

However, in spite of the presence of paternal plastids in the zygotes, strict maternal inheritance has been observed in lower plants and algae. Different mechanisms have been proposed that allow paternal plastid elimination from the mixed zygotes.

1- Methylation restriction system:

The enzymatic exclusion of paternal ctDNA was first proposed by Sager in Chlamydomonas, where two intact gametes of similar size fuse to form the zygote. She suggests that the ctDNA of the male gamete is enzymatically degraded, while the methylated ctDNA of the female gamete is protected. Methylation of specific sites of the female ctDNA occurs during gamete formation and presumably protects the DNA from digestion by a restriction endonuclease (Sager 1985). Mechanisms involved in the plastid inheritance of Chlamydomonas might conceivably occur in higher plants, although there is no evidence for this to date. Methylation of ctDNA in higher plants is infrequent, but may provide a mechanism for gene regulation in nonphotosynthetic plastids

in plant cells (Ngernprasirtsiri et al. 1988).

2-Active digestion model:

Kuriowa (1985) recently proposed active digestion of paternal ctDNA as a mechanism of maternal plastid inheritance in some alga. This hypothesis differs from the first one both by the type of nuclease and the mechanism of maternal ctDNA protection. An activated Nuclease C is hypothesized to digest the paternal ctDNA, while maternal ctDNA is protected by either a change in the membrane enclosing the female plastids or a change in the chloroplast matrix (Kuroiwa 1985). Specific mRNA synthesis occurs in the female nucleus after fusion, and its translation yields a regulatory protein which directly or indirectly activates Nuclease C. The preferentially-digested ct-nucleoids in the paternal plastid disappear, and the anucleate paternal chloroplast fuses with the protected maternal chloroplast in the zygote (Kuroiwa et al. 1985). Both of the previous models were proposed from studies in nonflowering plants, which are more easily studied than are flowering plants.

3- Multicopy model:

Gillham proposed the multicopy model as an alternative to the methylation restriction model (Gillham 1978). This model is based on the competitive exclusion of paternal plastid genomes, and resembles a model proposed to explain incompatible bacterial plasmid exclusion. A limited number of plastid genome attachment sites occur in the zygote, the

majority of which are preferentially occupied by maternal ctDNAs. Unattached genomes do not replicate, and are predominantly paternal (Gillham 1978). A better understanding of this process may result from cloning of the ctDNA replication origin sequence from both lower (Wu et al. 1986) and higher (Meeker et al. 1988) plants.

4- Vegetative segregation:

Birky (1978) suggests that where preferential plastid destruction occurs, as in the earlier mentioned models, it may not always be complete. Here a stochastic process of plastid sorting-out would ultimately determine the final plastid genotype of the developing zygote. If the paternal plastids in the early hetroplastidic zygote were present at low frequency, sorting-out during growth would ultimately yield maternal homoplastidic tissue. He further suggests that a similar stochastic sampling mechanism could be involved with uniparental inheritance in higher plants (Birky 1978). Mosaics and striped patterns of variegated plants that are plastid encoded are generally related to patterns of cell division (Kirk and Tilney-Bassett 1978).

MECHANISMS OF BIPARENTAL PLASTID INHERITANCE

Elimination of paternal plastids is not absolute even in plants that display high maternal transmission. Paternal chloroplast transmission in Nicotiana, a species normally

considered to exhibit strictly maternal inheritance, was demonstrated following antibiotic selection for a paternally encoded plastid resistance gene in tissue culture (Medgyesy et al. 1986). A very low frequency of paternal plastid transmission apparently occurs after sexual crosses in Epilobium (Schmitz and Kawallik 1986) and in Petunia hybrida Hort. (Cornu and Dulieu 1988), species previously considered to have strict maternal plastid transmission. Bulk analysis of reciprocal interspecific hybrid plant DNAs showed a very low frequency of paternal plastome transmission in Epilobium, when hybridized with a species-specific ctDNA fragment (Schmitz and Kawallik 1986). In Petunia, genotypes with an impaired mechanism for paternal plastid elimination had paternal plastids in 2% of their progeny (Cornu and Dulieu 1988). Even in the absence of this mechanism for paternal plastid elimination, maternal plastids still predominated relative to paternal plastids.

There is no single mechanism that explains the high paternal transmission of some plant species (Tilney-Bassett and Birky 1981; Smith et al. 1986). In all higher and lower plant species studied, the maternal plastid is present in the early zygote (Tilney-Bassett and Birky 1981; Russell 1987). Furthermore, plastid numbers do not differ significantly in egg cells of different Oenothera species that exhibit quite different frequencies of biparental inheritance (Chiu et al. 1988).

Variation in pollen plastid transmission may result from chloroplast load, lack of plastid replication, compartmentalization into tissues not contributing to the mature plant (Tilney-Bassett and Birky 1981), and differential destruction of plastids and/or their DNA (Sager 1985). Differential destruction after zygote formation seems unlikely in higher plants. Chiu et al. (1988) cites electron microscopic observations of Meyer and Stubbe which demonstrated that zygote development in Oenothera showed no indication of organelle degeneration from either parent following fertilization.

Variation in plastid load also may contribute to the efficiency of plastid transmission. If random sorting-out is hypothesized to function in determining paternal transmission of plastids during embryo development, the starting ratios of the two parental plastid types would contribute (Kirk and Tilney-Bassett 1978). Assuming equal starting plastid numbers, one hundred cell divisions are usually needed to complete conversion to homoplastidic tissue. However one or two cell divisions can result in complete sorting out of plastids in Pelargonium embryos (Tilney-Bassett and Birky 1981). They thus hypothesized that differences in plastid replication are important in determining which plastids are transmitted.

Chiu et al. (1988) analyzed the transmission abilities of four Oenothera plastid types in a constant nuclear background.

They found that different plastids had different intrinsic rates of multiplication. The faster replicating plastid in a mixed cell replicated at the expense of the slower one. The initiation time of multiplication was important also in plastid transmission. These effects were superimposed on an intrinsic maternal predominance. In plant species where paternal plastid transmission is predominant, multiplication efficiency need not be the only factor in determining the extent of paternal plastid transmission. Here, maternal plastid elimination might occur after fertilization through some active removal mechanism.

The overall volume of a given plastid type is believed closely related to its ctDNA content (Kuroiwa et al. 1988). Successive divisions without synthesis of plastid components and ctDNA during spermatogenesis probably accounts for their reduction in size and DNA content without affecting plastid number in the spores of some ferns (Kuroiwa et al. 1988). In general, plastids of the male parent contain fewer starch grains than do plastids of the female parent (Chiu et al. 1988). Presumably, the smaller plastids of male gametes are less efficient in transmitting their ctDNA than are the larger maternal plastids. However, three Oenothera plastid mutants had greater competitive abilities when contributed by the male parent than by the female parent (Chiu et al. 1988).

The first zygotic division is asymmetrical, and gives two cells of differing size (Cooper 1935; Dumas et al. 1984). The

large cell develops into the suspensor and the small cell develops into the main body of the embryo in Oenothera (Chiu et al. 1988) and Medicago (Cooper 1935). Embryos differentiate into two different organ systems, the axis and the cotyledons, during embryogenesis. The former gives rise to the root and the shoot. Paternal plastids might relocate to the large suspension cell, and thus not contribute to the progeny (Connett 1987). A similar mechanism might eliminate the clustered maternal plastids from the main body of the embryo, assuming that the location of the plastid cluster is maternally controlled, and thus result in a predominantly paternal plastid inheritance (Smith and Goldstein 1986; SE Smith personal communication).

Studies on plastid inheritance in somatic hybrid plants have improved our understanding of plastid inheritance in sexual progeny and of conversion to homoplasmy. Rapid sorting-out of plastids following protoplast fusion typically produces plants with only one or the other parental plastid types (Fluhr 1983). Probable factors influencing the sorting out of certain plastids include the physiological state of the parental plastids, the use of a genomic cytoplasmic selection procedure, and possible organelle-genome interrelationships. Nuclear-cytoplasmic incompatibility may function in some hybrids (Fluhr 1983).

The existence of two plastid types in the same cell does not typically result in recombination between their ctDNAs

(Chiu and Sears 1985). Revertants to heteroplasmy were produced by mutagen treatment in Chlamydomonas without any evidence of recombination (Spreitzer and Chastain 1987). Because these algae have only a single plastid containing 50-100 ctDNA copies per cell, heteroplasmy occurs within this single chloroplast. Heteroplasmy was stable through meiosis but mitotic segregation occurred (Spreitzer and Chastain 1987). However, recombination between ctDNAs has occurred in somatic hybrid tobacco plants produced under high selective pressure for the expression of both plastids (Medgyesy et al. 1985).

Unlike ctDNA, mitochondrial DNA (mtDNA) commonly shows recombination in somatic hybrids of dicotyledonous (D'hont et al. 1987; Rothenberg and Hanson 1988) and monocotyledonous (Ozias-Akins et al. 1988) plants. Unique mtDNAs different from those of either parental mtDNA occurred in somatic hybrids between Medicago sativa ssp. sativa and M. s. ssp. falcata (D'hont et al. 1987). A detailed study of a recombinant mitochondrial gene was reported in somatic hybrid cells of Petunia (Rothenberg and Hanson 1988). Their sequence data revealed recombination within a functional mitochondrial ATP synthase gene.

GENETIC CONTROL OF PLASTID INHERITANCE IN SEXUAL REPRODUCTION

The regulation of plastid transmission in plants is not

clearly understood. It is controlled by both nuclear and plastid genes in various species. There is no single mechanism that can account for plastid inheritance in all plant species (Connett 1987). The suggestion that various plant groups have different times and mechanisms of paternal plastid elimination during sexual reproduction implies that more than one gene is responsible. Genes related to the preferential destruction of male ctDNA function in the female cell nucleus of isogamous algae (having gametes of similar size, form, and structure, e. g. Chlamydomonas) but in the male cell nucleus of anisogamous algae (having gametes of different size, form, or structure, e. g. Bryopsis maxima) (Kuriowa 1986).

Studies using mutants that affect plastid transmission frequencies have helped to clarify the genetic control of plastid transmission. Nuclear mutation by ultraviolet light treatment prevented strict maternal chloroplast inheritance in the unicellular Chlamydomonas (Gillham et al. 1987). Plastid mutations which affect their transmission without affecting the transmission of mitochondria have been isolated (Gillham et al. 1987).

Plastid inheritance in higher plants is a complex process, and may be regulated by the female nuclear genome (Kirk and Tilney-Bassett 1978), the male nuclear genome (Miyamura et al. 1987; Cornu and Dulieu 1988), plastid genomes (Chiu et al. 1988), and/or combinations of these. The relative importance

of each component is unclear for most plant species. However, switching from biparental to maternal or vice versa is easily achieved in some plant species, and is therefore controlled by only a few genes (Kirk and Tilney-Bassett 1978).

Biparental plastid transmission is a good example of incomplete paternal plastid elimination in several plant genera; Pelargonium and Oenothera are most studied. Genes that control pollen transmission occur even in plants that exhibit strict maternal inheritance, such as the genus Petunia (Cornu and Dulieu 1988). One genotype of P. hybrida Hort. consistently shows low paternal plastid transmission when it is used as the male parent with different female genotypes (Cornu and Dulieu 1988). In contrast, mutant genotypes of plant species that usually show biparental plastid inheritance can show strict maternal inheritance (Vaughn 1981).

The maternal parent traditionally contributes much toward controlling biparental chloroplast inheritance in Pelargonium. A *Pr* gene in the female nucleus predominantly controls plastid inheritance patterns, presumably through its effect on plastid replication (Tilney-Bassett and Birky 1981; Tilney-Bassett and Abdel-Wahab 1982). Different distribution frequencies of maternal, biparental, or paternal plastid transmission result (Tilney-Bassett and Birky 1981). Two distinct segregation patterns occur with different female parents of Pelargonium. The first has a high maternal, intermediate biparental, and

low paternal transmission. The second has a high frequency of both maternal and paternal transmission but biparental plastid transmission is generally low (Tilney-Bassett and Birky 1981). Although the maternal nuclear genome clearly influences plastid transmission, plastids themselves have a role in their transmission. This is especially true in Oenothera, where it is widely accepted that the nuclear genome has a minor role (Chiu et al. 1988). Maternal plastids at early stages of zygote development have different rates of competitiveness than do plastids contributed by the paternal parent. The ability of a plastid to compete, as controlled by both nuclear (Tilney-Bassett et al. 1981) and plastid genes (Chiu et al. 1988), is a consequence of how soon and how rapidly it replicates. Plastids are generally transmitted at a higher rate in a cross when they are more closely related to the hybrid nuclear background than are the other plastids (Chiu et al. 1988). Three plastid classes in the subsection Euoneothera were identified based on their multiplication efficiency (Chiu et al. 1988).

Sequence homologies between higher plant and lower plant ctDNAs may in part control plastid inheritance. This implies that there is a common mechanism for parental plastid elimination acting in both higher and lower plants. Differences in plastid transmission between green and mutant plastids could result from the role of the plastome in its transmission (Chiu et al. 1988).

The effect of environment on plastid inheritance in higher plants is not well documented (Kirk and Tilney-Bassett 1978). However, nutritional stress may decrease the ability of the paternal parent to transmit plastid DNA in Chlamydomonas (Connett 1987). Mineral deficiency decreases chloroplast division in vegetative cells of higher plants (Daday et al. 1987). Environmental conditions can affect plastid division and replication. Light stimulates plastid division. Low light intensity stimulates chloroplast growth but suppresses their division (Daday et al. 1987).

CHLOROPLAST INHERITANCE IN ALFALFA

Lilienfeld (1962) first reported biparental inheritance in the genus Medicago in a study of nuclear-cytoplasmic incompatibility. Pronounced reciprocal differences in the behavior of plastids were noticed in crosses of two races of Medicago truncatula Gaertn. Reciprocal F_1 and back cross progeny showed evidence of paternal plastid transmission. Heteroplasmy was recognized in some of the hybrid embryos, and could be followed by somatic segregation of the plastids during development (Lilienfeld, 1962).

The first study of biparental inheritance in alfalfa (Medicago sativa ssp. sativa) was by Smith et al. (1986), who used reciprocal and other crosses between plants with normal

green and chlorophyll-deficient plastids. All developmental and genetic evidence indicated that both chlorophyll deficiencies represented plastid mutants. Development patterns of plastid segregation during somatic growth were typical of sorting out. The much higher transmission rate of both yellow-green and albino plastids via the pollen parent, as opposed to the seed parent, strongly suggested biparental chloroplast inheritance in alfalfa.

Biparental inheritance in this major crop species was unexpected, and provided a possible explanation for the hetroplastidic tissue seen in alfalfa. Rose et al. (1986) reported evidence for the existence of ctDNA heterogeneity in two protoplast donor plants through ctDNA restriction fragment analysis of 23 protoclonal lines. They concluded that plastid sorting out during vegetative propagation must have converted their donor plants to homoplasmy before analysis. Plastid sorting-out occurred following protoplast fusion of M. s. ssp. sativa and M. s. ssp. falcata (D'hont et al. 1987). The somatic hybrid plants contained only one of the two parental ctDNAs as judged by ethidium bromide fluorescence under ultraviolet light of the restricted ctDNAs, following electrophoretic separation of the fragments (D'hont et al. 1987).

Heteroplasmy was clearly established in green plants of several Medicago species (Johnson and Palmer 1989). Plastid DNAs from ten plants of three sativa subspecies (sativa,

varia, caerulea) were analyzed by restriction mapping and southern hybridization. Five plants were heteroplasmic. They suggested that pollen plastid transmission contributed to the heteroplasmy.

Recently, Lee et al. (1988) presented biochemical and cytological evidence conclusively establishing biparental inheritance in alfalfa. Using unique restriction fragment patterns of ctDNA from green and chlorophyll-deficient mutants, they demonstrated biparental plastid transmission by analyzing three sectorized plants from crosses between normal green plants and chlorophyll-deficient mutants. Hybridization of specific probes to southern blots of gels containing separately isolated ctDNAs from green and mutant sectors of these hybrid plants established the presence of both paternal ctDNAs.

The two types of plastids, mutant and green, were observed also in single cells in the mosaic tissue from one hybrid plant by transmission electron microscopy (Lee et al. 1988). The two chloroplast mutants differed from each other and the green chloroplasts both in their developmental patterns (Lee et al. 1989) and their transmission frequencies (Smith et al. 1986). Moreover, one of the two plastid mutants showed evidence of maternal nuclear genotypic influence on its transmission (Smith and Goldstein 1986). Parental genotypic influences on plastid transmission were suggested in other plant species where biparental inheritance occurs (Kirk and

Tilney-Bassett 1978).

Plastid inheritance in alfalfa follows a pattern similar to that observed in Pelargonium (Smith and Goldstein 1986). Results of crosses utilizing chlorophyll-deficient alfalfa mutants suggested that the predominant nuclear influence on plastid inheritance is maternal. Crosses of different genetic backgrounds showed apparent, but not definitive, differences in the frequency of plastid transmission.

Biparental inheritance of extranuclear organelles appears to be similar for both chloroplasts and mitochondria in alfalfa, although the latter are less studied due to the lack of observable markers. Fairbanks et al. (1988) documented biparental inheritance of mitochondria in alfalfa by showing that hybrid progenies inherited mitochondrial marker RNA molecules from either the maternal or paternal parents. It is unknown whether the inheritance of both organelles is controlled by the same mechanism or mechanisms in higher plants.

Cooper (1935) studied the macrosporogenesis and embryology of alfalfa and some other Medicago species microscopically. A large egg cell and two synergid cells were observed in the egg apparatus. The egg nucleus was in the basal region of the egg and was surrounded by dense cytoplasm containing numerous "starch grains". A layer of cytoplasm surrounded each of the two male gametes discharged from the male pollen tube in the vicinity of the egg. A recent study of alfalfa pollen showed

the presence of ctDNA in pollen generative cells (Corriveau and Coleman 1988).

The first zygotic division was unequal and resulted in an enlarged basal cell and a much smaller apical cell. Only the apical cell typically divided further, and formed a proembryo consisting of six cells which developed later into the true embryo (Cooper 1935). Smith and Goldstein (1986) proposed that plastid distribution following fertilization may explain biparental plastid inheritance in alfalfa.

Alfalfa is a good model system for studying plastid inheritance in higher plants. It is the only field crop that has biparental inheritance, and it can be readily regenerated from protoplasts. Physical maps of its plastid genome are available (Palmer et al. 1987; Johnson and Palmer, unpublished). Quantification of paternal inheritance in alfalfa may provide a better understanding of organelle inheritance in higher plants. Availability of chlorophyll deficiency plastid mutants in alfalfa will allow comparison of their transmission relative to green plastids, as well as facilitating any search for possible recombination. Alfalfa populations are highly heterogeneous, which should aid in the isolation of mutant plants unique in their plastid transmission pattern.

REFERENCES

Birky CW (Jr.) (1978) Transmission genetics of mitochondria

- and chloroplasts. *Annu Rev Genet* 12:471-512
- Birky CW (Jr.) (1983) Relaxed cellular controls and organelle heredity. *Science* 222:468-475
- Busby CH, Gunning BES (1988) Establishment of plastid-based quadriplarity in spore mother cells of the moss Funaria hygrometrica. *J Cell Sci* 91:117-126
- Chiu W, Sears BB (1985) Recombination between chloroplast DNAs does not occur in sexual crosses of Oenothera. *Mol Gen Genet* 198:525-528
- Chiu W, Stubbe W, Sears BB (1988) Plastid inheritance in Oenothera: organelle genome modifies the extent of biparental plastid transmission. *Curr Genet* 13:181-189
- Clauhs RP, Grun P (1977) Changes in plastid and mitochondrion content during maturation of generative cells of Solanum (Solanaceae). *Am J Bot* 64:377-383
- Connett MB (1987) Mechanisms of maternal inheritance of plastids and mitochondria: developmental and ultrastructural evidence. *Plant Mol Biol Rep* 4:193-205
- Cooper DC (1935) Macrosporogenesis and embryology of Medicago. *J Agric Res, Washington D.C.* 51:471-477
- Cornu A, Dulieu H (1988) Pollen transmission of plastid-DNA under genotypic control in Petunia hybrida Hort. *J Hered* 79:40-44
- Corriveau JL, Coleman AW (1988) Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 Angiosperm species. *Am J Bot* 75:1443-1458
- Daday HV, Lawrence M, Forrester RI, Whitecross MI, Possingham JF (1987) Nuclear DNA regulates the level of ribulose 1,5-bisphosphate carboxylase oxygenase in Medicago sativa L.. *Theor Appl Genet* 73:856-862
- Day A, Ellis TH (1984) Chloroplast DNA deletions associated with wheat plants regenerated from pollen: possible basis for maternal inheritance of chloroplasts. *Cell* 39:359-368
- D'hont A, Quetier F, Teoule E, and Dattee Y (1987) Mitochondrial and chloroplast DNA analysis of interspecific somatic hybrids of a Leguminosae: Medicago (alfalfa). *Plant Sci* 53:237-242
- Dumas C, Knox RB, McConchie CA, Russell SD (1984) Emerging physiological concepts in fertilization. *What's New Plant Physiol* 15:17-20
- Fairbanks DJ, Smith SE, Brown JK (1988) Inheritance of large mitochondrial RNA's in alfalfa. *Theor Appl Genet* 76:619-622
- Fluhr R (1983) The segregation of organelles and cytoplasmic traits in higher plant somatic fusion hybrids. In: Potrykus I, Harms CT, Hinnen A, Hutter R, King PJ, Shillito RD (eds) *Protoplasts. Lecture Proceedings*, Birkhauser Press, Basel, pp 85-92
- Gillham NW (1978) *Organelle heredity*. Raven Press, New York
- Gillham NW, Boynton JE, Johnson AM, Burkhart BD (1987) Mating type linked mutations which disrupt the uniparental

- transmission of chloroplast genes in Chlamydomonas. Genetics 115:677-684
- Goldberg RB (1988) Plants: novel developmental processes. Science 240:1460-1467
- Hatfield PM, Shoemaker RC, Palmer RG (1985) Maternal inheritance of chloroplast DNA within the genus Glycine, subgenus soja. J Hered 76:373-374
- Johnson LB, Palmer JD (1989) Heteroplasmy in chloroplast DNA in Medicago. Plant Mol Biol 12:3-11
- Johnson LB, Stuteville DL, Higgins RK, Skinner DZ (1981) Regeneration of alfalfa plants from protoplasts of selected Regen S clones. Plant Sci Lett 20:297-304
- Kirk JTO, Tilney-Bassett REA (1978) The Plastids. Their chemistry, Structure, Growth, and Inheritance, 2nd edn., Elsevier/North-Holland Biomedical Press, Netherlands
- Kuroiwa H, Sugai M, Kuroiwa T (1988) Behavior of chloroplasts and chloroplast nuclei during spermatogenesis in the fern, Pteris vittata L. Protoplasma 146:89-100
- Kuroiwa T (1985) Mechanisms of maternal inheritance of chloroplast DNA: an active digestion hypothesis. Microbiol Sci 2:267-270
- Kuroiwa T, Hori T (1986) Preferential digestion of male chloroplast nuclei and mitochondrial nuclei during gametogenesis of Bryopsis maxima Okamura. Protoplasma 133:85-87
- Lee DJ, Blake TK, Smith SE (1988) Biparental inheritance of chloroplast DNA and the existence of heteroplasmic cells in alfalfa. Theor Appl Genet 76:545-549
- Lee DJ, Blake TK, Smith SE, Bingham ET, Carroll TW (1989) Chloroplast genome mapping and plastid ultrastructure analysis of chlorophyll deficient mutants of alfalfa, Crop Sci 29:190-196
- Lilienfeld FA (1962) Plastid behavior in reciprocally different crosses between two races of Medicago truncatula Gaertn. Seiken Zihô 13:3-38
- Medgyesy P, Fejls E, Maliga P (1985) Interspecific chloroplast recombination in Nicotiana somatic hybrid. Proc Natl Acad Sci USA 82:6960-6964
- Medgyesy P, Páy A, Márton L (1986) Transmission of paternal chloroplast in Nicotiana. Mol Gen Genet 204:195-198
- Meeker R, Nielsen B, Tewari KK (1988) Localization of replication origins in pea chloroplast DNA. Mol Cell Biol 8:1216-1223
- Miyamura S, Kuroiwa T, Nagata T (1987) Disappearance of plastid and mitochondrial nucleoids during the formation of generative cells of higher plants revealed by fluorescence microscopy. Protoplasma 141:149-159
- Mogensen HL (1982) Double fertilization in barley and the cytological explanation for haploid embryo formation, embryoless caryopses, and ovule abortion. Carlsberg Res Commun 47:313-345
- Mogensen HL (1988) Exclusion of male mitochondria and plastids

- during syngamy in barley as a basis for maternal inheritance. Proc Natl Acad Sci USA 85:2594-2597
- Mogensen HL, Wagner VT (1987) Association among components of the male germ unit following in vitro pollination in barley. Protoplasma 138:161-172
- Mullet (1988) Chloroplast development and gene expression. Annu Rev Plant Physiol Plant Mol Biol 39:475-502
- Ngernprasirtsiri J, Kobayashi H, Akazawa T (1988) DNA methylation as a mechanism of transcriptional regulation in nonphotosynthetic plastids in plant cells. Proc Natl Acad Sci USA 85:4750-4754
- Oelmüller R, Levitan I, Bergfeld R, Rajasekhar VK, Mohr H (1986) Expression of nuclear genes as affected by treatments acting on the plastids. Planta 168:482-492
- Ozias-Akins P, Tabaeizadeh Z, Pring DR, Vasil IK (1988) Preferential amplification of mitochondrial DNA fragments in somatic hybrids of the Gramineae. Curr Genet 13:241-245
- Palmer JD, Osorio B, Aldrich J, Thompson WF (1987) Chloroplast DNA evolution among legumes: Loss of a large inverted repeat occurred prior to other sequence rearrangements. Curr Genet 11:275-286
- Rose RJ, Johnson LB, Kemble RJ (1986) Restriction endonuclease studies on the chloroplast and mitochondrial DNAs of alfalfa (Medicago sativa L.) protoclones. Plant Mol Biol 6:311-338
- Rothenberg M, Hanson MR (1988) A functional mitochondrial ATP synthase proteolipid gene produced by recombination of parental genes in Petunia somatic hybrid. Genetics 118:155-161
- Russell SD (1987) Quantitative cytology of the egg and central cell of Plumbago zeylanica and its impact on cytoplasmic inheritance patterns. Theor Appl Genet 74:693-699
- Sager R (1985) Chloroplast genetics. BioEssays 3:180-183
- Schmitz UK, Kawallik K (1986) Plastid inheritance in Epilobium. Curr Genet 11:1-5
- Sears BB (1980) Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. Plasmid 4:233-255
- Smith SE, Goldstein H (1986) Further investigation of organelle transmission in alfalfa. Report of the thirtieth North American Alfalfa Improvement Conference, page 46.
- Smith SE, Bingham ET, Fulton RW (1986) Transmission of chlorophyll deficiencies in Medicago sativa, evidence of biparental inheritance of plastids. J Hered 77:35-38
- Spreitzer RJ, Chastain CJ (1987) Heteroplasmic suppression of an amber mutation in the Chlamydomonas chloroplast gene that encodes the large subunit of ribulose biphosphate carboxylase/oxygenase. Curr Genet 11:611-616
- Sun G, Uyeda TQP, Kuroiwa T (1988) Destruction of organelle nuclei during spermatogenesis in Chara corallina examined by staining with DAPI and anti DNA antibody. Protoplasma 144:185-188
- Szmidt AE, Aldén T, Hällgren J (1987) Paternal inheritance of

- chloroplast DNA in Larix. Plant Mol Biol 9:59-64
- Tanaka I (1988) Isolation of generative cells and their protoplasts from pollen of Lilium longiflorum. Protoplasma 142:68-73
- Tilney-Bassett RAE, Abdel-Wahab OAL (1982) Irregular segregation at the Pr locus controlling plastid inheritance in Pelargonium: gametophytic lethal or incompatibility system? Theor Appl Genet 62:187-191
- Tilney-Bassett RAE, Birky CW (Jr) (1981) The mechanism of the mixed inheritance of chloroplast genes in Pelargonium. Evidence from gene frequency distributions among the progeny of crosses. Theor Appl Genet 60:43-53
- Vaughn K (1981) Organelle transmission in higher plants: organelle alteration vs. physical exclusion. J Hered 72:335-337
- Wagner DB, Furnier GR, Saghai-Maroo MA, Williams SM, Dancik BP, Allard RW (1987) Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc Natl Acad Sci USA 84:2097-2100
- Wu M, Lou JK, Chang CH (1986) Initiation of DNA replication in Chlamydomonas reinhardtii. In: RB Wickner, A Hinnebusch, AM Lambowitz, IC Gunsalus, and A Hollaender (eds.) Extrachromosomal elements in lower eukaryotes, Basic Life Science, Plenum Press, Vol 40, pp 67-80

CHAPTER TWO

HIGH TRANSMISSION OF PATERNAL PLASTID DNA IN ALFALFA PLANTS AS SHOWN BY RESTRICTION POLYMORPHIC ANALYSIS

INTRODUCTION

Lilienfeld (1962) first reported biparental chloroplast inheritance in the genus Medicago in a study of nuclear-cytoplasmic incompatibility. Pronounced reciprocal differences in plastid behavior were noted in crosses of two races of Medicago truncatula Gaertn. Heteroplasmy occurred in some hybrid embryos. Somatic segregation of the plastids often followed during development (Lilienfeld, 1962).

Biparental inheritance was reported in alfalfa (Medicago sativa ssp. sativa) following studies with plastid-encoded mutants for chlorophyll-deficiency (Smith et al. 1986) and plastid DNA (ctDNA) polymorphisms (Lee et al. 1988). Higher transmission rates of yellow-green and albino plastids via the pollen parent, as opposed to the seed parent, strongly suggested biparental chloroplast inheritance in alfalfa (Smith et al. 1986). Lee et al. (1988) confirmed this by demonstrating both heteroplasmicity and biparental plastid transmission in alfalfa, using ctDNA restriction fragment analysis and electron microscopy. Evidence came from three F₁ plants of crosses between a paternal parent possessing a

plastid-encoded chlorophyll deficiency mutant and a normal green maternal parent.

The potential of alfalfa for biparental plastid inheritance has been demonstrated by Corriveau and Coleman (1988), who detected plastid nucleoids in the pollen generative cells. Disappearance of plastid nucleoids from the generative cells may be responsible for maternal plastid inheritance in several higher plant species (Miyamura et al. 1987).

Heteroplasmy has been reported also in alfalfa with normal green chlorophyll, and presumably results from pollen plastid transmission. Rose et al. (1986) concluded that plastid sorting out during vegetative propagation must have converted two protoclone donor plants to homoplasmy probably before protoplast isolation. Johnson and Palmer (1989) clearly demonstrated heteroplasmy in several Medicago species by restriction fragment analysis. Five of 10 randomly selected plants, analyzed from populations of three accessions of M. sativa subspecies exhibiting ctDNA heterogeneity, were heteroplasmic.

It is not difficult to find ctDNA polymorphisms in alfalfa, even within a single accession, and restriction maps of mutant differences are available (Johnson and Palmer 1989, unpublished). Thus plastid transmission frequencies can be determined in reciprocal crosses, without concern for possible differences in competitiveness of plastids encoding their own chlorophyll deficiency. We report here high paternal and low

maternal plastid transmission in several alfalfa genotypes. The maternal nuclear genome, and possibly the paternal nuclear and/or plastid genome(s) as well, affected plastid transmission frequencies in these crosses.

MATERIALS AND METHODS

Parental plants and sexual crosses

Alfalfa plants representing two subspecies (ssp.) of Medicago sativa L. were used for crosses. Four plants of M. s. ssp. sativa ($2n=4x=32$) were: RS-K2C, a ramet of a plant (Johnson and Palmer 1989) selected from Regen S (Bingham et al. 1975), and MS-H, MS-I and MS-K, plants selected from P.I. 26590 obtained from the USDA Regional Plant Introduction Station, Pullman, WA. M. s. ssp. falcata plants were: two diploids ($2n=2x=16$); FA selected from P.I. 234815, and AN selected from 'Anik' (Pankiw and Siemens 1976). All plants were cloned by ramets before crossing in order to increase chances of plastid homozygosity. Parental plants were grown at 25°C either in growth chambers (16-hr photoperiod), or in a greenhouse with supplemental lighting at night in order to induce flowering in the winter. Most reciprocal crosses were made between plants at the same ploidy level. A schematic representation of these crosses is shown in Fig. 1. Since all of the parents

were self-fertile, they were emasculated by suction (Viands et al. 1988). Part of reciprocal cross number 6 was made by alternately tripping flowers of paternal and maternal parents with a toothpick, counting on the competitive advantage of heterologous pollen in alfalfa.

Planting and identification of hybrid plants

Seeds were harvested, scarified with sandpaper, and germinated in darkness on wet filter paper in petri plates at 21⁰C. Seedlings were planted individually in either 5 X 5 or 7 X 7 cm plastic pots. Plants from reciprocal crosses 2,3,4, and most plants of 1 were grown in growth chambers (16-hr photoperiod and 130 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ light intensity at 25⁰C) for 4 weeks before extraction. The slow-growing diploid hybrids of reciprocal cross 5 were similarly maintained for 8 weeks. Plants from reciprocal crosses 6,7, 8, and 9 and the remaining plants of reciprocal cross 1 were grown in the greenhouse with supplemental lighting, and were cut back several times before DNA extraction.

Four of five plants that showed only maternal ctDNA and about half of the plants in reciprocal crosses 1 to 3, were tested in order to establish whether they were hybrids by isozyme analysis (see below). Hybrid origins could be confirmed by either isozyme or ctDNA restriction patterns.

Hybrid plants from diploid-tetraploid crosses 6 through 9 were confirmed by their variegated flowers (hybrid phenotype), and by the faster growth of the tetraploid hybrid seedlings relative to the diploid seedlings produced by selfing. Tetraploidy was established for six of these diploid X tetraploid progeny using procedures of Snow (1963). High fertility following selfing makes it unlikely that any of the others were triploid, and necessitates the involvement of unreduced gametes (Pfeiffer and Bingham 1983) in their production.

Isozyme analysis

Alfalfa leaves (0.1-0.2 gm) were collected separately in microfuge tubes, and ground in liquid nitrogen. An equal volume of protein isolation buffer (100 mM Tris pH 8.5, 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2mM EDTA, 1% v/v 2-mercaptoethanol) was added to each microfuge tube before centrifuging at 15,000 g at 4°C for 10 min. Supernatants were transferred into new microfuge tubes containing 20 μl of loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol, 50% glycerol in isolation buffer). Protein extracts (50 μl) were loaded in high pH discontinuous vertical polyacrylamide gels (Hames 1981). Electrophoresis was in either 10% acrylamide gels for leucine aminopeptidases, or 7.5% acrylamide gels for esterases. Separations were

terminated, respectively, when the xylene cyanol dye or the bromophenol blue dye reached the end of the gel. Gel staining techniques were modified from Quiros (1981). Leucine aminopeptidase gels were equilibrated in 200 ml of cold 0.2 M Tris-HCl (pH 5.8) for 30 min, and transferred to 100 ml of the same buffer containing freshly combined fast blue B salt (50 mg) and L-leucine β -naphthylamide (50 mg, dissolved in 1 ml methanol). Staining was in the dark at 37°C for 1 hour. Esterase staining was in 50 ml of 0.2 M Tris-HCl (pH 7.1) containing freshly combined fast blue BB (75 mg) and α -naphthyl acetate (50 mg, dissolved in 5 ml acetone). Staining was at 21°C for 30 min.

DNA extraction and southern hybridization

Chloroplast DNAs of parents and a few hybrid plants were isolated from 10-30 gm of leaf tissue with the sucrose density gradient technique of Palmer (1986) with one modification; ethanol precipitation replaced dialysis. Total DNAs of single hybrid plants or their F₁ progeny were isolated from 200-300 mg leaf or root tissues (Rogers and Bendich 1985) after a 48-hr dark treatment. Random leaf samples were collected from large plants, but all trifoliolate leaves were harvested from seedlings. When required, DNA was quantified either spectrophotometrically or by comparison on test gels

containing known standards. DNA samples were digested by the restriction endonucleases *Bam* HI, *Hind* III, *Pst* I, or *Xba* I (Promega, Madison, WI) following manufacturer's instructions. Fragments were separated in 0.7% horizontal agarose gels (*Hind* III, *Pst* I, *Xba* I) or 1.1% gels (*Bam* HI) after Maniatis et al. (1982). Gels were stained in ethidium bromide and examined under ultraviolet light. Alkaline transfer of depurinated DNA to membranes (Zeta-ProbeTM, Bio-Rad, Richmond, CA; or ZetabindTM, AMF CUNO, Meriden, CT) was performed as published (Reed and Mann 1985). When a strong signal was needed, 1 M NaCl was used in the transfer buffer. Cloned alfalfa ctdNA fragments (Palmer et al. 1987) were used as probes to identify maternal and paternal origin (Rose et al. 1986; Johnson and Palmer 1989; Lee et al. 1989), and were labeled with [α ³²P] dCTP either by nick translation (Maniatis et al. 1982) or with an oligolabeling kit (Pharmacia, Piscataway, NJ). Unincorporated nucleotides were removed with a Sephadex G-50 spun column (Maniatis et al. 1982). Prehybridization and hybridization followed Zeta-ProbeTM-manufacturer's recommendations, but with 10% dextran sulphate added, for at least 6 hr. Filters were washed in solutions of increasing stringency up to 0.1x SSC and 1% SDS at 65°C for 30 min. Filters were exposed to X-ray film at -80°C for 4 to 96 hr with intensifying screens when needed.

RESULTS

Fragment length polymorphisms unique to each parental ctDNA were identified in individual plants (Table 1). The *Bam* HI polymorphisms shown in Table 1 are due to insertion/deletion events, while the polymorphisms with the other enzymes shown result from restriction site mutations (Johnson and Palmer 1989, unpublished). *Bam* HI restriction analysis was generally used where possible in order to eliminate any complications resulting from incomplete digestion. Exceptions were crosses two and five, which were tested by *Xba* I and *Pst* I respectively; the inter-subspecific crosses in which *Bam* HI and *Hind* III were used; and 12 plants from cross number one in which both *Bam* HI and *Xba* I were used.

A total of 212 hybrid progeny were analyzed for the parental source of their ctDNA. Plastid transmission was classified as paternal (P), biparental (BP), or maternal (M). Considering all crosses, only five hybrid plants showed no evidence of paternal ctDNA, while 81.6% of the plants had no detectable maternal ctDNA (Table 2). Isozyme analysis of four of the five hybrids (the fifth did not survive after DNA extraction) with maternally-derived plastids showed that all were hybrids and not the result of selfing. The extent of plastid mixing in the 34 hybrid heteroplastidic (BP) plants averaged 59% paternal based on estimates of banding intensity,

and ranged from predominantly paternal to predominantly maternal. The sensitivity for detection of a minor parental ctDNA population varied among autoradiograms. However, less than 1% of a minor ctDNA was detected in an in vitro mixture of two different ctDNAs (data not shown), a limit similar to the 0.1% value of Scowcroft and Larkin (1981).

Heteroplasmy was observed in hybrid plants from 13 of the 19 crosses (Table 2). Examples are shown in Fig. 2. The top growth of several heteroplastidic plants was removed and allowed to regrow from the crown after the first ctDNA analysis. The new shoots were analyzed in groups of two or one. Results of analysis of one heteroplastidic plant are shown in Fig. 2A and 2B. Homoplastidic maternal, homoplastidic paternal, and heteroplastidic shoots arose from the same crown. Analysis of regrowth from a few heteroplastidic plants showed slight changes in ctDNA ratios, presumably as a result of sorting out (Fig. 2D). However, progeny from cross number one showed no change in the number of paternal, biparental, and maternal plants, when extracts from a later cutting were compared with initial extracts. CtDNA analysis of F_1 progeny from five hybrid plants, three of them heteroplastidic, all exhibited expected restriction patterns (not shown). Also, roots from eight homoplastidic plants from different reciprocal crosses all contained the same plastid type as did their shoots (not shown).

Table 2 breaks down ctDNA transmission into paternal (P), biparental (BP), and maternal (M) classes for all crosses, as summarized by both frequency of plants/class and percentage of plants. Frequencies of M were very low, making comparisons difficult. Because % M is small, the percentages of P and BP complement each other. The frequency of progeny exhibiting only P ranged from 37% to 100% with different crosses, with the 100% values occurring only where few plants were analyzed.

The percentage of P plants was compared among all combinations of the three-plant reciprocal crossing scheme (reciprocal crosses 1, 2, and 3), where more plants were analyzed (Table 2). A higher % paternal transmission was noted in crosses 1-b and 3-b where the MS-K plant was used as a female parent (90 and 93%), than when it was used as a male parent in crosses 1-a and 3-a (76 and 80%). With RS-K2C and MS-H, the % paternal transmission was 77% in either direction. This distinction was not obvious when crosses were analyzed by comparing the % of paternal ctDNA in the progeny.

Intra-subspecific reciprocal crosses were more similar to each other in paternal transmission (81-86% P) than they were to inter-subspecific reciprocal crosses (42-67% P) (Table 3). When inter-subspecific crosses were compared, female and male tetraploid parents had a higher % P transmission than did the diploid parents. Small sample sizes, in part due to the low success rate of interploidy crosses through unreduced gametes, made comparisons within an individual reciprocal cross

meaningless. Thus data were pooled. Paternal plastid transmission followed two patterns. Intra-subspecific crosses showed a high % P and low % PB in the progeny. Inter-subspecific crosses exhibited a relatively low % P and high % PB in the hybrids.

DISCUSSION

Our ctDNA analysis clearly establishes the role of pollen transmission of plastids in the initiation of heteroplasmy (Fig. 2A-D), a phenomenon noted in randomly selected green Medicago plants by Johnson and Palmer (1989). As might be expected, plastids in heteroplastidic plants can sort out, resulting in homoplastidic tissues (Fig. 2B). Eight homoplastidic plants possessing paternal plastids were analyzed. All showed the same plastid type in both root and shoot. Clearly, the high frequency of paternal homoplasmy is not the result of any preferential sorting out in the embryo during early cell divisions that give rise to root:shoot differentiation.

Maternal nuclear control of plastid transmission frequency seems apparent in our three-plant reciprocal crossing scheme (crosses 1, 2, and 3 in Fig. 1, and Table 1), where three parents are each reciprocally crossed with the other two. Crosses 1-b and 3-b involving MS-K as the maternal parent show a higher paternal plastid transmission than do the other four

crosses, where MS-H or RS-K2C are the maternal parents. The percentage of plants showing biparental transmission is correspondingly reduced. Maternal nuclear regulation appears to explain these data. Regulation by paternal or maternal plastids is not involved in this difference, since the same plastids, when transmitted to other nuclear backgrounds, are all equally successful. A plastid which solely regulates its own high transmission rate should increase its transmission frequency in either direction in a reciprocal cross, which was not the case here. However, plastid influences on their own transmission are well documented in other species such as Oenothera (Chiu et al. 1988). For our material, the hybrid nuclear genotype also cannot be a major factor, again because of differences in plastid transmission frequency within a reciprocal cross. It appears that MS-K, when used as a female parent, transmitted its plastids at a lower rate than did either MS-H or MS-K as maternal parents. The differences between nuclear genotypes is obscured when total paternal ctDNA in the progeny is estimated, presumably because during sorting out in the BP class the minor (maternal) ctDNA is frequently lost (Birky 1983; Kirk and Tilney-Bassett 1978).

The maternal parent contributes much toward controlling biparental chloroplast inheritance in Pelargonium. A *Pr* gene in the female nucleus predominantly controls plastid inheritance patterns, presumably through its effect on plastid

replication (Tilney-Bassett and Birky 1981; Tilney-Bassett and Abdel-Wahab 1982). Two different distribution frequencies of maternal, biparental, or paternal plastid transmission result with different female parents of Pelargonium (Tilney-Bassett and Birky 1981). The first has a high maternal, intermediate biparental, and low paternal transmission. The second has a high frequency of both maternal and paternal transmission but biparental plastid transmission is generally low (Tilney-Bassett and Birky 1981). Both patterns are different than in alfalfa, where paternal transmission is high, and maternal low.

Two observations are of interest in the inter-subspecific crosses (Table 3). First, our tetraploid M. s. ssp. sativa plants, used as either male or female parents, transmitted their plastids at a higher rate than did the diploid M. s. ssp. falcata genotypes. Plastid load may contribute to the efficiency of plastid transmission (Kirk and Tilney-Bassett 1978). Plastid number in diploid cells, which are typically smaller, should be lower than that in larger tetraploid cells (Molin et al. 1982; Pyke and Leech 1987). However, our hybrid plants produced by these crosses were all tetraploids, indicating fertilization by unreduced gametes, which are of similar size (Pfeiffer and Bingham 1983) and presumably equal proplastid number to tetraploid gametes. This would suggest that more than plastid load is involved.

Second, in both inter-subspecific reciprocal crosses, a

higher % BP and a lower % P occurred relative to the intra-subspecific crosses. Genetic differences between the two subspecies may influence plastid transmission frequencies in crosses between them. These subspecies, while sexually compatible, are considered by some to be distinct species (Lesins and Lesins 1979). The genetic control of plastid transmission in these crosses is difficult to evaluate, in part because of pooling of crossing data necessitated by the small sample size. However, it appears that factors other than the maternal nuclear genotype contribute to the control of plastid transmission. For example, plant RS-K2C was used as a female parent in four different crosses. Paternal plastid transmission ranged from 37 to 100%. Differences are apparent even though sample size for some of these crosses was small (Table 1). Presumably paternal nuclear and/or plastid factor(s) also are involved. For whatever reason, a high frequency of heteroplastidic plants results.

Smith et al. (1986) evaluated transmission frequencies with chlorophyll-deficient plastid mutants in reciprocal crosses. Their transmission frequencies are difficult to compare with ours because of differences in the parental material and the scoring procedure, although they also saw a high frequency of paternal transmission. Recovery of the chlorophyll deficiency used in either parent in the progeny was an indicator of chloroplast transmission by that parent. However, in our data this is equivalent to the summation of biparental transmission

frequency, and paternal or maternal frequency, depending on the direction of the cross.

Extracts from several alfalfa plants were tested with two restriction enzymes for evidence of ctDNA recombination. As might be expected (Chiu and Sears 1985), none was found when 12 hybrid plants of cross one (RS-K2C X MSK) were compared following separate digestions with *Bam* HI and *Xba* I. The two sites are located almost opposite each other on the circular alfalfa plastid genome (Johnson and Palmer 1989) and the parent ctDNAs differ at both sites. To our knowledge, recombination between two ctDNAs has been demonstrated only in a somatic hybrid tobacco plant produced under high selection pressure for the expression of both plastids (Medgyesy et al. 1985). A much more extensive study would be required to detect recombination in our material, and should probably concentrate on progeny derived from selfing heteroplastidic plants.

Biparental plastid inheritance has been previously demonstrated in both conifers (Szmidt et al. 1987; Wagner et al. 1987) and alfalfa (Lee et al. 1988) by various DNA restriction techniques. The use of ctDNA as a marker for plastid inheritance, when combined with controlled crosses, allows a more meaningful estimation of the frequency of transmission than does the use of chlorophyll deficiency mutants, which may be transmitted at a competitive

disadvantage. In Pelargonium, green plastids were transmitted at a higher rate than were mutant plastids (Tilney-Bassett and Birky 1981; Tilney-Bassett and Abdel-Wahab 1982). Two chloroplast mutants of Oenothera exhibited reduced transmission from the female parent relative to a third mutant, which was transmitted similarly to the wild type (Chiu et al. 1988).

There are other advantages as well for using ctDNA restriction fragment length polymorphisms for plastid transmission studies. A relatively large number of reciprocal crosses can be analyzed without loss of sensitivity for detection of a minor plastid component in a tissue. Crosses are not limited to genotypes that differ in plastid-encoded chlorophyll deficiency mutants. Both chloroplast and leucoplast analyses are possible. Aberrant transmission ratios due to seedling death resulting from chlorophyll deficiency mutants are eliminated. Induction and reversion of plastid mutations by nuclear mutator genes are common, and may interfere with plastid inheritance studies when chlorophyll deficiency markers are used (Gillham 1978; Cornu and Dulieu 1988).

Extraction of total plant DNA, followed by southern hybridization, allows analysis of small seedlings, and is much more sensitive than is ethidium bromide ctDNA staining. Comparative studies of plastid transmission involving both chlorophyll deficiency and ctDNA restriction pattern markers

are not available.

We are unaware of other studies where plastid transmission frequency and vegetative segregation have been evaluated by ctdNA restriction analysis with green tissues rather than with plastid-encoded chlorophyll deficiency mutants. The high plastid paternal transmission observed with alfalfa, similar in some ways to Pelargonium, suggests the potential of this species for studies on the regulation of plastid transmission through the pollen.

REFERENCES

- Bingham ET, Hurley LV, Kaatz DM, Saunders JW (1975) Breeding alfalfa which regenerates from callus tissue in culture. *Crop Sci* 15:719-721
- Chiu W, Sears BB (1985) Recombination between chloroplast DNAs does not occur in sexual crosses of *Oenothera*. *Mol Gen Genet* 198:525-528
- Chiu W, Stubbe W, Sears BB (1988) Plastid inheritance in *Oenothera*: organelle genome modifies the extent of biparental plastid transmission. *Curr Genet* 13:181-189
- Cornu A, Dulieu H (1988) Pollen transmission of plastid-DNA under genotypic control in Petunia hybrida Hort. *J Hered* 79:40-44
- Corriveau JL, Coleman AW (1988) Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 Angiosperm species. *Am J Bot* 75:1443-1458
- D'hont A, Quetier F, Teoule E, and Dattee Y (1987) Mitochondrial and chloroplast DNA analysis of interspecific somatic hybrids of a Leguminosae: Medicago (alfalfa). *Plant Sci* 53:237-242
- Fairbanks DJ, Smith SE, Brown JK (1988) Inheritance of large mitochondrial RNA's in alfalfa. *Theor Appl Genet* 76:619-622
- Johnson LB, Palmer JD (1989) Heteroplasmy in chloroplast DNA in Medicago. *Plant Mol Biol* 12:3-11
- Hames BD (1981) An introduction to polyacrylamide gel electrophoresis. In: Hames BD and Rickwood D (Eds.). *Gel Electrophoresis, a practical approach, Practical Approaches Series*, IRL Press, Oxford Washington DC. pp 1-86

- Kirk JTO, Tilney-Bassett REA (1978) The Plastids. Their chemistry, structure, growth, and inheritance, 2nd edn., Elsevier/North-Holland Biomedical Press, Netherlands
- Lee DJ, Blake TK, Smith SE (1988) Biparental inheritance of chloroplast DNA and the existence of heteroplasmic cells in alfalfa. *Theor Appl Genet* 76:545-549
- Lee DJ, Blake TK, Smith SE, Bingham ET, Carroll TW (1989) Chloroplast genome mapping and plastid ultrastructure analysis of chlorophyll deficient mutants of alfalfa. *Crop Sci* 29:190-196
- Lesins KA, Lesins I (1979) Genus Medicago (Leguminosae), A taxogenetic study. W Junk, The Hague
- Lilienfeld FA (1962) Plastid behavior in reciprocally different crosses between two races of Medicago truncatula Gaertn. *Seiken Zihô* 13:3-38
- Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. Cold Spring Harbor Press, Cold Spring Harbor, NY
- Medgyesy P, Fejís E, Maliga P (1985) Interspecific chloroplast recombination in a Nicotiana somatic hybrid. *Proc Natl Acad Sci USA* 82:6960-6964
- Miyamura S, Kuroiwa T, Nagata T (1987) Disappearance of plastid and mitochondrial nucleoids during the formation of generative cells of higher plants revealed by fluorescence microscopy. *Protoplasma* 141:149-159
- Molin WT, Meyers SP, Baer GR, Schrader LE (1982) Ploidy effects in isogenic populations of alfalfa. II. Photosynthesis, chloroplast number, ribulose 1,5-bisphosphate carboxylase, chlorophyll, and DNA in protoplasts. *Plant Physiol* 70:1710-1714
- Palmer JD (1986) Isolation and structural analysis of chloroplast DNA. *Methods Enzymol* 118:167-186
- Palmer JD, Osorio B, Aldrich J, Thompson WF (1987) Chloroplast DNA evolution among legumes: Loss of a large inverted repeat occurred prior to other sequence rearrangements. *Curr Genet* 11:275-286
- Pankiw P, Siemens B (1976) Anik alfalfa. *Can J Plant Sci* 56:203-205
- Pfeiffer TW, Bingham ET (1983) Abnormal meiosis in alfalfa, Medicago sativa: cytology of 2N egg and 4N pollen formation. *Can J Genet Cytol* 25:107-112
- Pyke KA, Leech RM (1987) The control of chloroplast number in wheat mesophyll cells. *Planta* 170:416-420
- Quiros CF (1981) Starch gel electrophoresis technique used with alfalfa and other Medicago species. *Can J Plant Sci* 61:745-749
- Reed KC, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res* 13(20):7207-7221
- Rogers SO, Bendich AG (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol Biol* 5:69-76
- Rose RJ, Johnson LB, Kemble RJ (1986) Restriction endonuclease studies on the chloroplast and mitochondrial DNAs of

- alfalfa (Medicago sativa L.) protoclones. Plant Mol Biol 6:311-338
- Scowcroft WR, Larkin PJ (1981) Chloroplast DNA assortments randomly in intraspecific somatic hybrids of Nicotiana debneyi. Theor Appl Genet 60:179-184
- Sears BB (1980) Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. Plasmid 4:233-255
- Smith SE, Bingham ET, Fulton RW (1986) Transmission of chlorophyll deficiencies in Medicago sativa, evidence of biparental inheritance of plastids. J Hered 77:35-38
- Snow R (1963) Alcoholic hydrochloric acid-carmines as a stain for chromosomes in squash preparations. Stain Technol 38:9-13
- Szmidt AE, Aldén T, Hällgren J (1987) Paternal inheritance of chloroplast DNA in Larix. Plant Mol Biol 9:59-64
- Tilney-Bassett RAE, Abdel-Wahab OAL (1982) Irregular segregation at the *Pr* locus controlling plastid inheritance in Pelargonium: gametophytic lethal or incompatibility system? Theor Appl Genet 62:187-191
- Tilney-Bassett RAE, Birky CW (Jr) (1981) The mechanism of the mixed inheritance of chloroplast genes in Pelargonium. Evidence from gene frequency distributions among the progeny of crosses. Theor Appl Genet 60:43-53
- Viands DR, Sun P, Barnes DK (1988) Pollination control: mechanical and sterility. In: Hanson AA, Barnes DK, Hill RR (eds). Alfalfa and alfalfa improvement. Number 29 in the series Agronomy. Am Soc Agron. Crop Sci Soc Am. and Soil Sci Soc Am. Madison, pp 931-960
- Wagner DB, Furnier GR, Saghai-Marooof MA, Williams SM, Dancik BP, Allard RW (1987) Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc Natl Acad Sci USA 84:2097-2100

Table 1. Restriction fragment length polymorphisms (in kbp) used to distinguish parental ctDNAs following restriction enzyme digestion and hybridization with alfalfa ctDNA fragments

Parents	Restriction enzyme / fragment ^a					
	<u>Sam</u> HI ^b / 12.5	<u>Hind</u> III ^c / 12.5	<u>Pst</u> I ^c / 18	<u>Xba</u> I ^d / 6.2		
MS-K	2.00	10.0	18.0		7.8, 0.5	
MS-I	2.00	10.0	18.0		7.8, 0.5	
MS-H	1.90	10.0	18.0		7.8, 0.5	
RS-K2C	1.85	10.0	18.0		8.3	
AN	1.75	6.0, 4.0	10.0, 8.0		8.3	
FA	1.70	6.0, 4.0	18.0		8.3	

^a Cloned alfalfa ctDNA probes (6.2, 12.5, and 18 kbp pst I fragments) provided by J. D. Palmer (Univ. Michigan, Ann Arbor). A restriction map is published (Palmer *et al.* 1987). Note that the 12.5 kbp pst I fragment was initially estimated at 12.7 kbp (Johnson and Palmer 1989).

^b These polymorphisms occur within the hypervariable region reported and mapped by Johnson and Palmer (1989). Sizes estimated to nearest 0.05 kbp. Apparently corresponds to polymorphisms seen by Lee *et al.* (1989).

^c Johnson and Palmer (unpublished) observed Medicago sativa ssp. sativa and M. sativa ssp. falcata ctDNA differences here.

^d Polymorphisms first observed here by Rose *et al.* (1986). See Johnson and Palmer (1989) for a restriction map.

Table 2. Single and reciprocal crosses used for evaluating ctDNA transmission of different alfalfa genotypes

Cross No.	♀ parent	♂ parent	Plants/Class		Total analyzed	Transmission (%/Class)			Total paternal ctDNA in progeny (%) ^d	
			P ^a	BP ^b		P	BP	M		
1-a	RS-K2C	MS-K	28	7	37	76	19	5	88	
1-b	MS-K	RS-K2C	45	4	50	90	8	2	93	
2-a	RS-K2C	MS-H	10	3	13	77	23	0	90	
2-b	MS-H	RS-K2C	10	2	13	77	15	8	83	
3-a	MS-H	MS-K	16	4	20	80	20	0	95	
3-b	MS-K	MS-H	27	1	29	93	3	3	96	
4-a	RS-K2C	MS-I	5	0	5	100	0	0	100	
4-b	MS-I	RS-K2C	4	1	5	80	20	0	92	
5-a	AN	FA	12	2	14	86	14	0	91	
5-b	FA	AN	7	1	8	87	13	0	99	
6-a	RS-K2C	FA	3	5	8	37	63	0	75	
6-b	FA	RS-K2C	2	2	4	50	50	0	70	
7-a	MS-K	AN	1	1	2	50	50	0	75	
7-b	AN	MS-K	1	0	1	100	0	0	100	
8	MS-H	AN	1	1	2	50	50	0	95	
9	AN	RS-K2C	1	0	1	100	0	0	100	
Total			173	34	5	212	82	16	2	91

^a Paternal

^b Biparental

^c Maternal

^d Transmission was estimated by the following formula:
 $\% \text{ paternal ctDNA transmission} = \frac{P}{P + [BP \times p\text{-ctDNA}]}$

where p-ctDNA equals the mean proportion of paternal ctDNA in BP plants as visually estimated from southern blots (mean=0.59 paternal, data not shown).

Table 3. Summary of crosses within and between two Medicago sativa subspecies

Parents	Plants/Class			Total analyzed	Transmission (%/Class)			Total paternal ctdNA in progeny (%) ^d
	P ^a	Bp ^b	M ^c		P	Bp	M	
Total reciprocal crosses within <u>ssp. sativa</u>	145	22	5	172	84	13	3	92
Total reciprocal crosses within <u>ssp. falcata</u>	19	3	0	22	86	14	0	94
♀ <u>sativa</u> x ♂ <u>falcata</u>	5	7	0	12	42	58	0	79
♀ <u>falcata</u> x ♂ <u>sativa</u>	4	2	0	6	67	33	0	80
Total	173	34	5	212	82	16	2	91

^{a, b, c, d} Same as in **Table 1.**

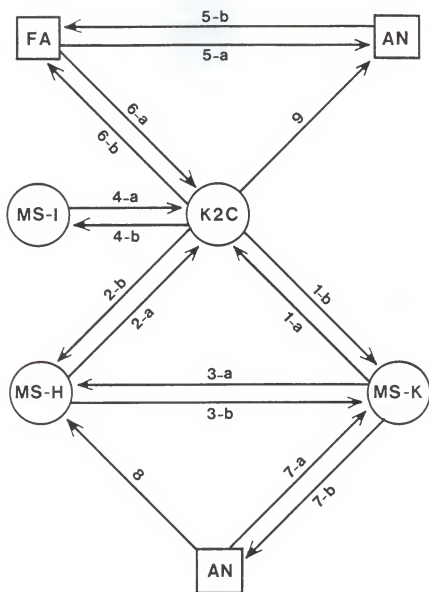


Fig. 1. A schematic representation of all sexual crosses made for evaluating paternal plastid transmission. Circles represent tetraploid parents (*Medicago sativa* ssp. *sativa*), squares represent diploid parents (*M. sativa* ssp. *falcata*). The arrows represent crosses. Tails start from the pollen parent. Heads point to the maternal parents. The crosses are numbered on or under the arrows. See Materials and Methods for an explanation of the plant genotypes.

Fig. 2A-D. Autoradiograms showing ctDNA transmission in alfalfa sexual crosses, and conversion of heteroplasmy to homoplasmy during vegetative propagation. A: *Bam* HI-digested DNAs from leaves of four hybrid seedlings of RS-K2C X MS-K, separated in a 1.1% agarose gel and hybridized to the 12.5 kbp alfalfa ctDNA probe. Hybrids in lanes 1, 3, and 4 show a 2.00 kbp fragment from the MS-K paternal plastids, while the heteroplastidic hybrid in lane 2 has fragments from both paternal and maternal (1.85 kbp) plastids. B: *Bam* HI-digested DNA, of regrowth shoots from the hybrid in lane 2 above separated in a 1.1% agarose gel and hybridized to the 12.5 kbp alfalfa ctDNA probe. Leaves from three different sectors, each containing 1 or 2 shoots regrown from the crown, were separately analyzed for their plastid source. Sectors in lanes 2 and 3 show maternal and paternal plastids respectively. The sectors in lane 1 have not yet sorted out. C: *Xba* I-digested DNA extracted from seven RS-K2C X MS-H hybrids, separated in a 0.7% agarose gel and hybridized to the 6.2 kbp alfalfa ctDNA probe. Lane 2 contains a heteroplastidic plant with 7.8 and 8.3 kbp fragments. All other lanes have plants with only paternal plastids, as shown by the presence of 7.8 kbp fragments. D: *Hind* III-digested DNA from two plants from a FA X RS-K2C at two consecutive harvests, separated in a 0.7% agarose gel and hybridized to the 12.5 kbp alfalfa ctDNA probe. The maternal FA ctDNA contains an additional restriction site, producing 6.0 and 4.0 kbp fragments instead of a 10 kbp fragment. Lanes 1 and 2 contain extracts from a heteroplastidic hybrid, and show decreased maternal ctDNA and increased paternal ctDNA in the later extract (lane 2). Only paternal ctDNA was detected in extracts of the hybrid plant represented in lanes 3 and 4.

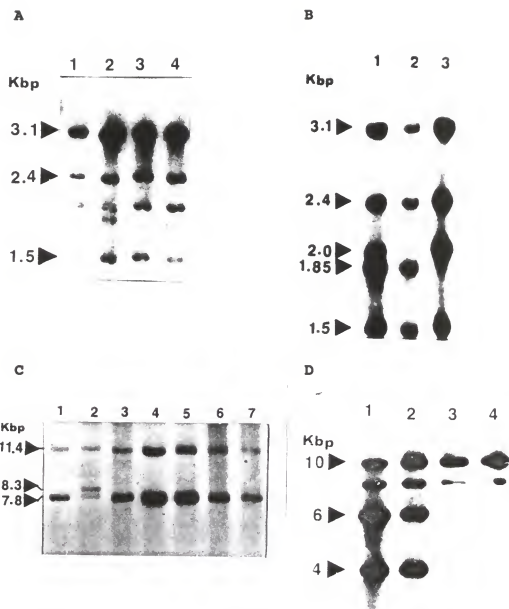


Fig. 2A-D.

HIGH TRANSMISSION OF PATERNAL PLASTID DNA IN ALFALFA PLANTS
AS SHOWN BY RESTRICTION POLYMORPHIC ANALYSIS

by

SAMEER AHMAD MASOUD

B.S., University of Jordan
Amman, Jordan, 1982

AN ABSTRACT OF MASTER'S THESIS

submitted in partial fulfillment of the
requirements for the degree
MASTER OF SCIENCE

Department of Plant Pathology
KANSAS STATE UNIVERSITY
Manhattan, Kansas

1989

SUMMARY

A high frequency of biparental plastid transmission was demonstrated in progeny from crosses among normal green alfalfa plants. Plastid transmission was analyzed by hybridization of radiolabeled alfalfa plastid DNA (ctDNA) probes to southern blots of restriction digests of the progeny DNA. Each probe revealed a specific polymorphism differentiating the parental plastid genomes. Thirty-four of 212 progeny were heteroplastidic, with their ctDNAs ranging from predominantly paternal to predominantly maternal. Regrowth of shoots from heteroplasmic plants following removal of top growth revealed the persistence of mixed plastids in a given plant. However, different shoots within a green heteroplasmic plant exhibited paternal, maternal, or mixed ctDNAs. Evidence of maternal nuclear genomic influence on the frequency of paternal plastid transmission was observed in reciprocal crosses. F_1 Progeny from tetraploid ($2n=4x=32$) Medicago sativa ssp. sativa X diploid ($2n=2x=16$) M. s. ssp. falcata crosses were tetraploid, and resulted from unreduced gametes. Although progeny were few, results suggested that more than the maternal genome alone functions in controlling plastid transmission. Only five of 212 progeny ctDNAs lacked evidence of a definitive paternal fragment.